Pestic. Sci. 1997, **51**, 1–6

Mode of Action of β-Carboline Convulsants on the Insect Nervous System and Their Potential as Insecticides

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(Received 28 June 1996; revised version received 5 January 1997; accepted 26 March 1997)

Abstract: Little information is available on the actions of β-carboline convulsants on insect GABA receptors or their potential as insecticides. Accordingly, two compounds (3-ethoxy-β-carboline, 3-EBC; dimethoxy-β-carboline-3-methyl ester, DMCM) were studied for their effects on *Drosophila melanogaster* larval neuron discharge and also in lethality bioassays on adult female *D. melanogaster* and adult male *Blattella germanica*. Recordings of nerve spiking in the isolated larval central nervous system showed that 3-EBC and DMCM inhibited nerve discharge, and this inhibitory effect was not additive with that of GABA, confirming that the inhibition was expressed through an action on the GABA receptor. Nerve blockage induced by β-carbolines could not be reversed by picrotoxinin, indicating that there may exist some overlap or negative allosteric coupling between the picrotoxinin and β-carboline binding sites. DMCM and 3-EBC effectively antagonized the effects of exogenously applied GABA in nerve preparations from insecticide-susceptible larvae. In contrast, preparations from the *rdl* strain of *D. melanogaster*, which possesses a GABA receptor that is highly resistant to cyclodienes and related convulsants, were less sensitive to the GABA antagonist effect of DMCM. Neither of the β-carbolines produced any appreciable mortality in insects, even when synergized with piperonyl butoxide or *S*,*S*,*S*-tributyl phosphorotrithioate, The toxicity of the β-carbolines is probably limited by their relatively weak effects on the GABA receptor and perhaps also by pharmacokinetic factors. These considerations, coupled with the cross-resistance observed in cyclodiene-resistant insects, suggest that the currently available β-carbolines are not viable as lead compounds for insecticide screening efforts.

Pestic Sci., **51**, 1–6, 1997
No. of Figures: 5. No. of Tables: 1. No. of Refs: 15

Key words: GABA receptor, GABA antagonist, insecticide resistance, β-carboline convulsants, insect nerve

1 INTRODUCTION

Inhibitory synapses in insects and mammals have proven to be sensitive sites of action for neurotoxic insecticides, where the target protein is the $GABA_A$ receptor/chloride channel complex.[1] This type of GABA receptor responds to the binding of the neurotransmitter, 4-aminobutyric acid (GABA), by activating a chloride ion channel.[2,3] Several chemical classes of convulsants and commercial insecticides, including lindane, endosulfan and fipronil, exert their toxic action by stabilizing non-conducting states of the chloride ion channel.[1,4] The $GABA_A$ receptor also possesses a binding site for the benzodiazepines, which enhance

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Contract grant sponsor: US Department of Agriculture; Contract grant number: 6129430.

 Pestic. Sci. 0031-613X/97/$17.50. Printed in Great Britain

GABAergic transmission by potentiating the action of GABA and are epitomized by the anxiolytics diazepam and chlordiazepoxide.[2] In contrast, convulsant or proconvulsant β-carbolines act as inverse agonists at the benzodiazepine binding site on the mammalian $GABA_A$ receptor. That is, they bind to the benzodiazepine site on the receptor complex, but instead of potentiating the action of GABA, they decrease its ability to activate the chloride channel.[2,3] Because there is continuing interest in developing insecticides directed against the insect GABA receptor, and because there are known differences in pharmacology between insect and mammalian GABA receptors that might be exploited for selective insect control,[5] the present study was undertaken in order to characterize the mode of action and toxicity of β-carbolines in insects. A preliminary report of these results has appeared.[6]

2 EXPERIMENTAL METHODS

2.1 Insects

All insects used in these studies were maintained in culture at the Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA. The two strains of *Drosophila melanogaster* Meig. used were a common wild-type strain (*Oregon-R*) that was insecticide-susceptible, and a cyclodiene-resistant strain (*rdl*) carrying a mutation in a GABA receptor subunit.[4] Both strains were reared on commercial diet (Carolina Biological Supply, Burlington, North Carolina, USA). Adult male German cockroaches, *Blattella germanica* (L.), were maintained on dry dog food and water, *ad libitum*.

2.2 Chemicals

Picrotoxinin (PTX) was purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Piperonyl butoxide (PB) was a gift from FMC, Princeton, New Jersey, USA and *S,S,S*-tributyl phosphorotrithioate (DEF) was kindly provided by J. Ottea, Louisiana State University, Baton Rouge, Louisiana, USA.

2.3 Synthesis of 3-ethoxy-β-carboline (3-EBC) and dimethoxy-β-carboline-3-methyl ester (DMCM)

Because the synthesis and structural confirmation of 3-EBC and DMCM were accomplished several years ago and follow well-established procedures,[7–9] only a

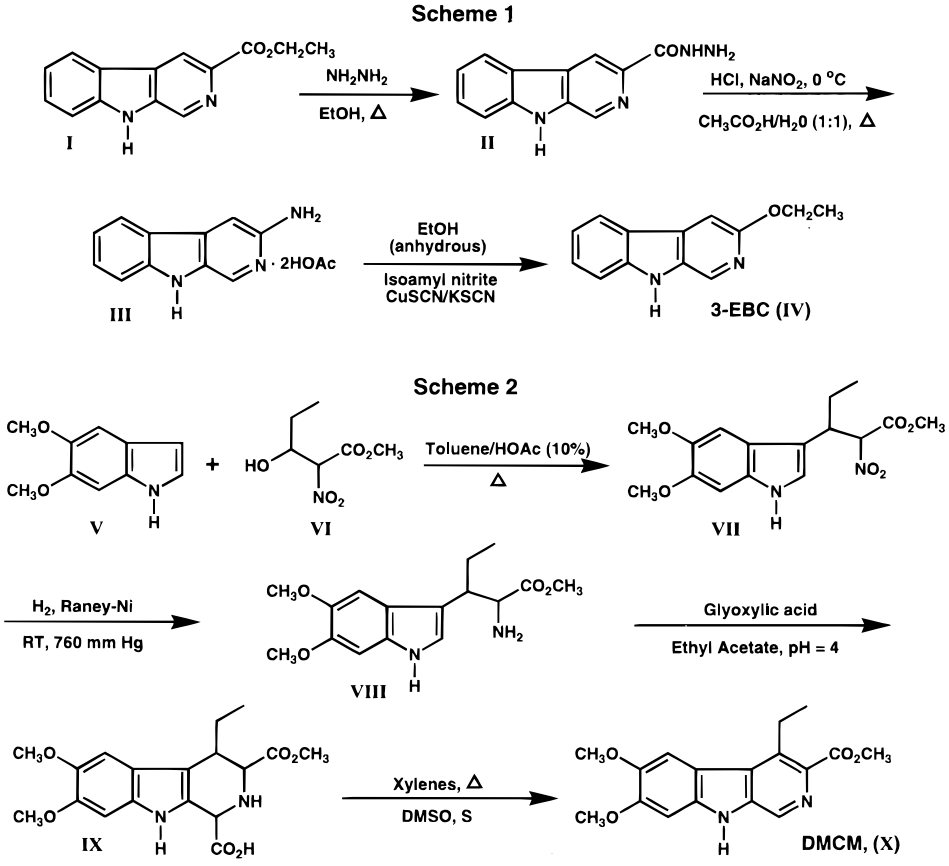

Fig. 1. Chemical syntheses of 3-ethoxy-β-carboline (3-EBC, Scheme 1, top) and dimethoxy-β-carboline-3-methyl ester (DMCM, Scheme 2, bottom).

brief description will be given here. To obtain 3-EBC (Fig. 1, Scheme 1), β-carboline-3-carboxylic ethyl ester (**I**) was treated with 98% hydrazine in the presence of ethanol at reflux to provide the corresponding carbohydrazide (**II**) in excellent yield. The carbohydrazide was dissolved by the dropwise addition of concentrated hydrochloric acid at 0°C followed by addition of sodium nitrite. Curtins rearrangement of the intermediate azide compound (not shown) was accomplished in acetic acid + water (1 + 1 by volume) at reflux to yield 3-amino-β-carboline as the diacetate salt (**III**). Treatment of 3-amino-β-carboline with anhydrous ethanol in the presence of isoamyl nitrite, CuSCN and KSCN provided 3-EBC (**IV**) in good yield. DMCM was prepared in the following manner (Fig. 1, Scheme 2). Treatment of 5,6-dimethoxyindole (**V**) with 3-hydroxy-2-nitro-5-oxa-heptanoic acid methyl ester (**VI**) in the presence of 10% acetic acid/toluene at reflux provided the corresponding indole (**VII**) in good yield. Raney nickel reduction of this nitro intermediate yielded the amino methyl ester (**VIII**) in excellent yield. A modified Pictet-Spengler reaction employing glyoxylic acid in ethyl acetate at pH 4 provided the tetrahydro β-carboline (**IX**). Subsequent decarboxylation in xylenes at reflux followed by oxidation using elemental sulfur in DMSO at 140°C resulted in DMCM (**X**).

2.4 Neurophysiological studies

Neurophysiological recordings were performed on third-instar larvae of *D. melanogaster* as originally described.[10] Briefly, suction electrode recordings were made from any convenient groups of peripheral nerves on an isolated, transected larval central nervous system (CNS). Electrical activity emanating from the CNS was digitized at 10 Hz to convert normal burst discharges into single or small groups of spikes in order to simplify the data analysis. Recordings were time-compressed and analyzed on a microcomputer-based physiological recording system (MacLab™, AD Instruments, Milford, Massachusetts, USA). Following dissection, nerve activity was monitored for 3–7 min to establish a baseline, and then the preparation was treated by application of compounds directly to the bath. Mixing was accomplished by gentle pipetting. β-Carbolines were dissolved in DMSO and the final concentration of vehicle in the bath never exceeded 0·2%. GABA was dissolved in physiological saline. Inhibition of nerve firing by β-carbolines alone was determined after a 10-min incubation period (Fig. 2). To assess the effect of β-carbolines on GABA-dependent inhibition, susceptible or insecticide-resistant preparations were exposed to 1 mM GABA, and once activity had declined to a stable level, the CNS was challenged with β-carboline. Responses were quantitated by summing suprathreshold nerve bursts for a 3-min period immediately after β-carboline treatment. Dose-response data were analyzed by computer-generated fit to a sigmoid curve using InPlot™ and statistical analysis was performed using InStat™ (both from GraphPad Software, San Diego, California, USA).

2.5 Mortality bioassays

Lethality of β-carbolines was tested by topical application to adult male German cockroaches and in a feeding bioassay on adult female *D. melanogaster* (*Oregon-R*). In feeding assays, both mixed function oxidase (PB) and esterase (DEF) synergists were employed in an effort to reveal the intrinsic lethality of the β-carbolines. Compounds were dissolved in either methanol or ethanol. Treatment solutions containing toxicant and synergist in solvent were formulated by adding 5-μl aliquots to 0·5 ml of a 100 g litre^{-1} sucrose solution. The treated sucrose solution was then applied to a cotton dental wick, which was used to stopper glass vials containing 10 adult female flies. Synergists were used at final concentrations of 3400 mg kg^{-1} (PB) and 1700 mg kg^{-1} (DEF), while 3-EBC and DMCM were tested at maximal concentrations of 50 and

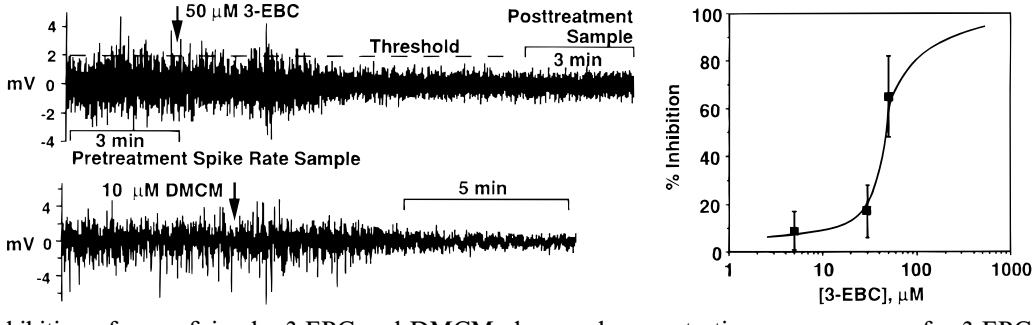

Fig. 2. Slow inhibition of nerve firing by 3-EBC and DMCM alone and concentration-response curve for 3-EBC. In this and in Figs 3 and 5, treatment of the preparation occurred at the arrows, and compounds were continuously present at the indicated concentration from that point onward. For quantitation of inhibitory effect, the initial firing rate was determined over a 3-min period prior to treatment with 3-EBC. An identical post-treatment sample was taken after a 10-min incubation and percentage inhibition was calculated from these two values. Points on the graph are the mean percentage inhibition (±SEM), with three to seven replicates per concentration.

100 mg kg^{-1}, respectively. Mortality was scored after 24 or 48 h, and corrected for control mortality by Abbott's formula.[11]

3 RESULTS

When applied alone, the β-carbolines inhibited the firing of larval nerve preparations and, unlike GABA, this inhibition was insensitive to PTX. Both 3-EBC and DMCM typically induced a slow blockage of nerve firing that was complete within 10 min after treatment (Fig. 2). Comparison of pre- and post-treatment firing rates with various concentrations of 3-EBC showed a steep dose-response relationship, and the inhibition was incomplete, even at 50 μM (Fig. 2). This concentration was at the limit of solubility, so higher amounts of 3-EBC could not be used. Computer analysis yielded a good fit to a sigmoid curve ($r^2 = 0{\cdot}984$), and gave a calculated IC_{50} value of 43 μM with 95% confidence limits of 16 to 116 μM, if a maximal inhibition of 100% was assumed. Dose-response studies were not attempted with DMCM. Additional experiments demonstrated that, unlike GABA, neuronal inhibition by the β-carbolines was not antagonized by PTX (Fig. 3). PTX reversed GABA-dependent inhibition of CNS discharge within 1 min, but showed no effect on 3-EBC blockade for at least 5 min after treatment.

Because high concentrations of 3-EBC were required to block nerve activity and because the blockage was insensitive to PTX, experiments were performed to test whether the inhibition was actually caused by an action on the GABA receptor, as opposed to a local anesthetic-type effect. To this end, treatments of 3-EBC and GABA were applied alone or simultaneously in order to test for additivity of effect (Fig. 4). Analysis of inhibition was performed at two different times following treatment, and in all cases simultaneous exposure to both inhibitors gave less than the expected 100% inhibition. Additional preliminary experiments performed on

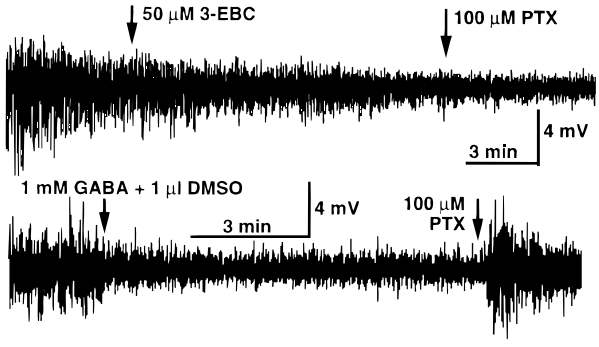

Fig. 3. Extent of PTX-dependent antagonism of nerve blockage by 3-EBC and GABA. DMSO was applied with GABA so that both experiments contained the same total amount of solvent, since DMSO was the vehicle used to apply both 3-EBC and PTX.

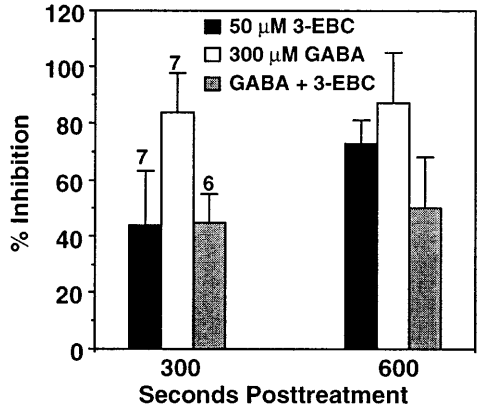

Fig. 4. Mean percentage inhibition of nerve firing by GABA, 3-EBC and both compounds applied simultaneously. For each experiment, inhibition was determined 300 and 600 s after treatment. Numbers above the standard error bars indicate the number of replicates in each experimental group.

housefly larval sensory nerves supported these results, since 50 μM 3-EBC had no effect on nerve spiking in sensory preparations (data not shown).

The β-carbolines were also tested for their ability to reverse the effects of pre-applied GABA in both insecticide-susceptible and cyclodiene-resistant CNS preparations. In susceptible preparations, DMCM (Fig. 5A) and 3-EBC (Fig. 5B) rapidly reversed the inhibitory effect of 1 mM GABA. In contrast, preparations from cyclodiene-resistant larvae showed a consistent delay of at least 5 min before firing resumed (Fig. 5C). The nerve firing in susceptible preparations exposed to DMCM averaged 162($\pm$66) bursts within a 3-min period immediately following treatment, while in resistant preparations the average response was 6·8($\pm$3·1) bursts. The difference was statistically significant ($P < 0{\cdot}036$, Mann-Whitney test).

Although the β-carbolines affected nerve discharge rates *in vitro*, and showed some ability to antagonize GABA, they displayed virtually no lethality to insects (Table 1). Topical applications were applied at the

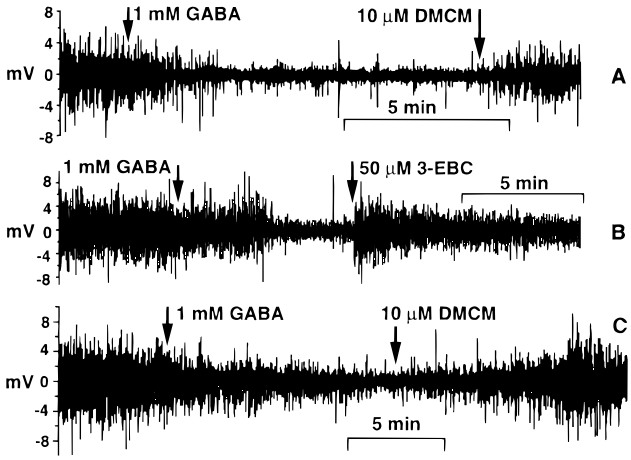

Fig. 5. Antagonism of GABA-dependent inhibition by β-carbolines in (A and B) susceptible and (C) cyclodiene-resistant preparations.

TABLE 1
Toxicity of DMCM and 3-EBC to Insects in 48-h Bioassays

Treatment	*Insect*	*Compound*	*Dose or Dosage*	*Synergist*	*n*[a]	*Mortality* (%)
Topical	*B. germanica*	3-EBC	40 μg per insect	—	7	19
Topical	*B. germanica*	DMCM	40 μg per insect	—	10	29
Diet	*D. melanogaster*	3-EBC	50 mg kg^{-1}	PB	40	0
Diet	*D. melanogaster*	DMCM	100 mg kg^{-1}	PB	30	17
Diet	*D. melanogaster*	DMCM	100 mg kg^{-1}	DEF	30	0

[a] Number of insects used.

limits of solubility of the β-carbolines, and using the highest amount of solvent the cockroaches could tolerate. These conditions allowed for doses of *c*. 900 mg kg^{-1}, using an observed typical body weight of about 45 mg for adult male *B. germanica*. In spite of these high doses, the mortality from 3-EBC or DMCM treatment did not exceed 29% (Table 1). Similarly, high concentrations of β-carbolines in the diet caused little mortality against adult female *D. melanogaster*, even in the presence of the mixed-function oxidase inhibitor PB (Table 1). Additional studies with the esterase inhibitor DEF and DMCM observed no mortality during 48-h diet exposures. No studies with DEF and 3-EBC were attempted, since 3-EBC lacks an ester moiety (Fig. 1).

4 DISCUSSION

In this study, β-carbolines displayed some effects consistent with their known actions on mammalian neurons, but the results have also revealed some unique aspects of their effects in insects. The β-carbolines antagonized the inhibitory effects of exogenously applied GABA, consistent with their known ability to inhibit GABA-induced currents in voltage-clamped mammalian[12,13] and insect[14] neurons. However, the overall effect of β-carbolines on nerve discharge rates was inhibitory. This reduction in nerve firing was unexpected and suggested a weak activation of the GABA receptor that needs to be confirmed with more detailed electrophysiological analysis. This effect may be similar to the ability of the avermectins to activate a chloride channel conductance while blocking the effect of exogenously applied GABA.[15] Combination treatments with 3-EBC and GABA were not additive, suggesting a negative allosteric coupling between their binding sites, and confirming that the β-carboline effect was expressed *via* interaction with the GABA receptor. A local anesthetic type of effect was ruled out by the lack of effect on sensory nerve discharge. Studies with mammalian neurons[12] found that high concentrations of β-carbolines could potentiate GABA-induced currents, and although such an action could explain a β-carboline-induced inhibition of nerve firing, it is not consistent with the results of our additivity studies (Fig. 4).

Our studies with PTX antagonism and cross-resistance in a cyclodiene-resistant strain of *D. melanogaster* revealed other unique features of the actions of β-carbolines in insects. Inhibition of nerve firing caused by 3-EBC was insensitive to subsequent challenge with PTX, which suggests some overlap or negative allosteric coupling between the binding sites for these two compounds. Consistent with this conclusion is the observation that CNS preparations from cyclodiene-resistant *D. melanogaster* larvae show cross-resistance to the GABA antagonism of DMCM. In contrast, studies in embryonic kidney cells expressing GABA receptors formed by mammalian α_1 and β_1 subunits showed that these receptors were sensitive to PTX, but not to DMCM or another benzodiazepine inverse agonist, 4′-chlorodiazepam.[13] Sensitivity to DMCM and 4′-chlorodiazepam, in addition to PTX, was achieved by the additional expression of the γ_2 subunit. From these results, the authors concluded that the inverse agonists were not acting isosterically with PTX.[13] It would be interesting to see whether homo-oligomeric *rdl* receptors expressed in *Xenopus* oocytes display resistance to β-carbolines. If resistance were observed, it would confirm a genuine difference in the actions of β-carbolines in mammals and insects.

The currently available β-carbolines appear to be poor leads for insecticide screening and synthesis efforts. These compounds caused little or no mortality in either topical or diet bioasssays, even when tested at high levels in the presence of synergists. In addition, they did not display high potency on nerve preparations *in vitro*. However, no dose-response experiments were performed with DMCM, which appeared to be more active than 3-EBC. Finally, the cross-resistance to DMCM in *rdl* CNS preparations suggests the presence of some level of cross-resistance to β-carbolines in field populations of insects.

5 ACKNOWLEDGMENTS

This material is based upon work supported by the Cooperative State Research Service, US Department of

Agriculture, under project No. 6129430. Any opinions, findings, conclusions or recommendations expressed are those of the authors and do not necessarily reflect the view of the US Department of Agriculture.

REFERENCES

1. Bloomquist, J. R., *Comp. Biochem. Physiol.*, **106C** (1993) 301–14.
2. Kerr, D. I. B. & Ong, J., *Med. Res. Rev.*, **12** (1992) 593–636.
3. Schwartz, R. D., *Biochem. Pharmacol.*, **37** (1988) 3369–75.
4. Zhang, H-G., ffrench-Constant, R. H. & Jackson, M. B., *J. Physiol.*, **479** (1994) 65–75.
5. Rauh, J. J., Lummis, S. C. R. & Sattelle, D. B., *Trends Neurosci.*, **11** (1990) 325–9.
6. Bloomquist, J. R., LaLoggia, A., Reddy, M. & Cook, J., *Options 2000, Eighth IUPAC International Congress of Pesticide Chemistry*, International Union of Pure and Applied Chemistry/American Chemical Society, Washington DC, 1994, p. 216.
7. Cain, M., Weber, R., Guzman, F., Cook, J., Barker, S., Rice, K., Crawley, J., Paul, S. & Skolnik, P., *J. Med. Chem.*, **25** (1982) 1081–91.
8. Allen, M. S., Hagen, T. J., Trudell, M. L., Codding, P. W., Skolnick, P. & Cook, J. M., *J. Med. Chem.*, **31** (1988) 1851–61.
9. Neel, G., Eder, U., Huth, A., Rahtz, D., Schmiechen, R. & Seidelmann, D., *Heterocycles*, **20** (1983) 1295–313.
10. Bloomquist, J. R., ffrench-Constant, R. H. & Roush, R. T., *Pestic. Sci.*, **32** (1991) 463–9.
11. Abbott, W. S., *J. Econ. Ent.*, **18** (1925) 265–7.
12. Im, H. K., Im, W. B., Carter, D. B. & McKinley, D. D., *Br. J. Pharmacol.*, **114** (1995) 1040–4.
13. Puia, G., Santi, M., Vicini, S., Pritchett, D., Seeburg, P. & Costa, E., *Proc. Natl Acad. Sci. USA*, **86** (1989) 7275–9.
14. Von Keyserlingk, H. C. & Willis, R. J., *Neurotox '91: Molecular Basis of Drug and Pesticide Action*, ed. I. Duce. Elsevier Applied Science, London, 1992, pp. 79–104.
15. Duce, I. R. & Scott, R. H., *Br. J. Pharmacol.*, **85** (1985) 395–401.